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A Mutagenesis Approach for the Study of the Structure-Function Relationship of Human Immunodeficiency Virus Type 1 (HIV-1) Vpr

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1. Introduction

Before the era of molecular biology, the methods available for an understanding of gene function were limited. Such studies typically relied on the ability to identify and isolate naturally occurring variants exhibiting a defect in function. Hence, the progress was slow in this regard. The discoveries in the field of microbiology, combined with advances in technology in the later part of 20th century, dramatically changed this scenario. The current molecular biological techniques enable site-directed mutagenesis approaches for generating a gene with a specific amino acid substitution, mutation, or a deletion or for truncating a gene anywhere in a matter of days.

The present review highlights the studies conducted on human immunodeficiency virus type 1 (HIV-1) Vpr, an auxiliary protein associated with virus particles. Vpr contains 96 amino acids, and it is a multifunctional protein. To analyze the contribution of specific residues to protein function and to the cytopathic effects in HIV-1-infected individuals, investigators from several laboratories, including ours, took advantage of a novel approach. Specifically, this approach involved exchange of residues through mutagenesis. The choice of residue was based on the information available regarding the naturally occurring polymorphisms at the level of individual amino acids in Vpr. The results from these studies support a link between polymorphisms in these genes and the disease status of infected individuals, who are known as progressors or non-progressors. In addition the studies have shed light on the structure-function relationship of Vpr.

The Joint United Nations Program on HIV/AIDS (UNAIDS) (2010) reports that the worldwide prevalence of those living with human immunodeficiency virus (HIV) is between 31-35 million as of the end of 2009. Roughly 2.6 million new cases of HIV infection occurred in the same year. That number has likely remained approximately constant.

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Although the most common route of HIV infection is via sexual contact, the use of contaminated drug paraphernalia, mother-child transmission via pregnancy or breastfeeding, and tainted blood transfusions comprise other means of infection.

The symptomatic outcome of infection is AIDS, usually occurring ~10 years after initial infection. CD4+ T-cell counts drop below 200, and subsequent severe immune dysfunction results. This eventually leads to fatal coinfection by opportunistic pathogens. The advent of highly active retroviral therapy (HAART) in the 1990s led to a drastically improved prognosis for AIDS patients (Peters and Conway, 2011). This triple-drug cocktail controls viremia and allows immune function to recover to nearly uninfected levels, with the caveat of near-perfect patient adherence to a difficult combination of drug regimens. The extremely rigid treatment schedules have resulted in low compliance, leading to the emergence of viruses that exhibit resistance to drugs.

2. Genetic organization of HIV

The causative agents of AIDS have been identified as human immunodeficiency virus types 1 (HIV-1) and 2 (HIV-2). Both HIV-1 and HIV-2 are members of the lentivirus family of retroviruses. HIV-1 is the predominant virus responsible for AIDS throughout the world. The schematic representation of the genome organization of HIV-1 is shown in **Figure 1.** The genome of HIV-1 codes for two regulatory proteins (Tat and Rev) and four auxiliary proteins (Vif, Vpr Vpu and Nef), in addition to structural proteins Gag, Gag-Pol and Env. The genome organization of HIV-2 is similar to HIV-1. The unique genes are *vpu* and *vpx* for HIV-1 and HIV-2, respectively. With respect to viral morphogenetic events, the lentiviruses are similar to alpharetroviruses, gammaretroviruses and deltaretroviruses. During virus infection, Gag and Gag-Pol proteins are synthesized in the cytoplasm and transported to the cell membrane, where virus assembly occurs. In the case of HIV-1, the non-structural proteins Vpr, Vif, and Nef are also packaged into the virus particles.

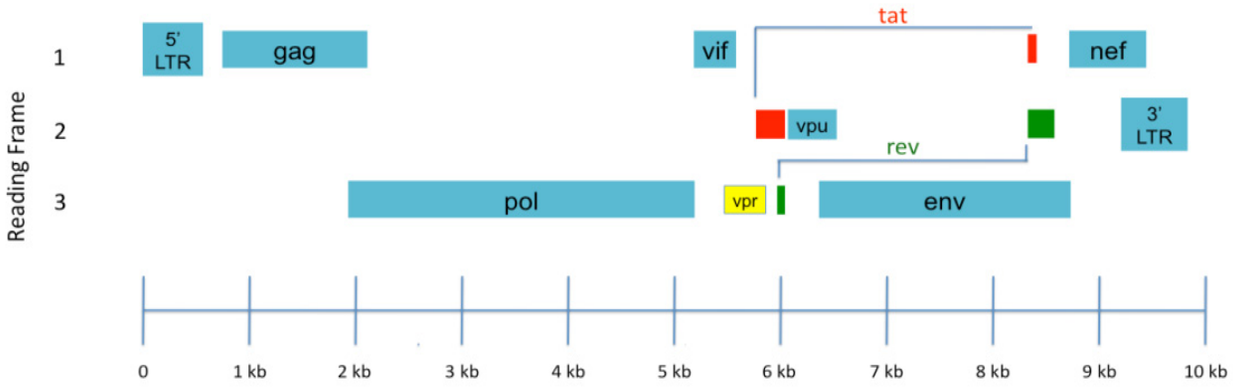

Fig. 1. Organization of the HIV-1 Genome.

3. Heterogeneity in the human immunodeficiency virus

Within HIV-1-positive patients, the high error rate of reverse transcription can produce many variants, or quasispecies. After seroconversion to HIV-positive status, the viral loads measure at 10000-50000 viral RNA copies per ml of patient sera during the asymptomatic

stage; in the later stages of disease, viral RNA genomes can increase to several million copies per ml (Poropatich and Sullivan, 2011; Tungaturthi et al., 2004). Thus, a high replication rate produces tremendous variation in the viral population within a single patient. The pressures of the host immune response drive the selection of variants from early stages of infection, until the host immune response cannot cope with the viral diversity (Fischer et al., 2010; Wolinsky et al., 1996).

Studies of polymorphisms in a number of the genes of HIV-1 confirm the propensity of the virus to escape host immunity (Fischer et al., 2010; Fischer et al., 2007; Gaschen et al., 2002; Korber et al., 2001). As expected, the antigenic Env protein exhibits the highest mutability. Comparisons of current strains of HIV show a staggering 20% variability within a subtype and up to 35% variability between subtypes (Gaschen et al., 2002). To highlight the problem of this variability in vaccine development, the evolutionary dynamics of influenza virus provides a revealing picture. The influenza genome varies by 1-2% per year, enabling influenza virus escape from polyclonal vaccine responses and necessitating annual vaccine changes (Fischer et al., 2007; Gaschen et al., 2002). Along with intra- and inter-subtype variation in HIV, *env* shows on average a 10% change in genetic diversity over the course of an infection in a single patient (Korber et al., 2001). In addition to the *env* gene, Korber et al. (2001) estimated the percent variation in sequences culled from an HIV-1 database compared to the HXB2 strain consensus in *tat* and *gag*. They found 9% and 5% variation, respectively. From another study, *gag* and *pol* remained relatively conserved; but the rest of the genes exhibited high variability comparable to that of *env* (Gaschen et al., 2002) **(Figure 1)**. The vast genetic diversity seen in these studies thus far exemplifies the major obstacle to vaccine development.

The variation in the viral genome throughout the population potentially correlates with the variation among HIV-infected individuals. In the progressor group, in which AIDS develops 10 years after initial infection, CD4+ T-cell counts (the primary marker of AIDS progression) generally fall below 200 cells per µl and causing loss of cell-mediated immunity (Levy, 2009). Along with the normal progressors, several other categories of disease progression exist. Long term non-progressors (LTNPs) and elite controllers (EC) do not receive HAART and do not show the aforementioned clinical signs of AIDS for up to 20 years after infection. CD4+ T-cell counts are maintained to levels above 350 cells per µl and viral loads to under 2000 copies per ml. Viral loads in these groups can range as low as 50-2000 copies per ml (Poropatich and Sullivan, 2011). In rapid progressors (RP), CD4+ T-cell counts generally decline 3-8 years postinfection. Although individual host genetics likely play a role in differential disease progression (Goulder et al., 1997), the presence of multiple quasispecies in patients potentially explains the differences in disease outcome.

4. Polymorphisms in specific HIV-1 genes

Several genes of HIV have a functional role in disease symptoms (Caly et al., 2008; Casartelli et al., 2003; Tolstrup et al., 2006). As Nef downregulates MHC class I antigen presentation involved in generating a cytotoxic T-lymphocyte (CTL) response, polymorphisms in this gene correlate with the LTNP group (Caly et al., 2008; Casartelli et al., 2003; Tolstrup et al., 2006). Similarly the role of Tat as a transactivator of the HIV-1 promoter in the long-terminal-repeat (LTR) region and its genetic heterogeneity suggest a functional role for Tat in disease progression and outcome (Bratanich et al., 1998; Korber et al., 2001). Tat can

upregulate LTR-transcriptional activity and thus replication. Cellular and viral transcription increases several hundredfold (Irish et al., 2009) in the presence of Tat. Various Tat proteins isolated from different subtypes show differences in the ability to induce viral and cellular gene transcription (Roof et al., 2002).

The multifunctional Viral Protein R (Vpr), although labeled as an accessory protein, has a vital role in efficient replication of the virus in the non-dividing macrophages and monocytes (Balliet et al., 1994; Connor et al., 1995). Vpr interacts with the Gag protein for packaging into the virion. It remains bound to the preintegration complex (PIC) upon entry of the viral contents into the target cell during infection, most likely aiding PIC entry into the nucleus. It has a highly functional role in replication as a transactivator of the LTR, binding to a number of host transcription factors, such as Sp1 (Sawaya et al., 1998) and TFIIB (Agostini et al., 1999). Extensive studies have shown that coactivation of glucocorticoid receptor by Vpr, resulting in transcription of elements in the promoter regions of the HIV LTR and host genes, enhances viral replication and downregulates host immune responses (Ayyavoo et al., 1997; Hapgood and Tomasicchio, 2010). Vpr also drives apoptosis and G2 cell cycle arrest (Morellet et al., 2009; Pandey et al., 2009; Tungaturthi et al., 2004). The processes enhance immune escape and HIV-1 replication, respectively. As Vpr seems to be essential in the viral life cycle, it is likely that *vpr* gene sequences will exhibit signature changes among the categories of HIV-1 disease progression. Due to the importance of Vpr, this review focuses mainly on the polymorphisms in *vpr*, their effects on Vpr function, and their potential consequences on disease outcome. The review will also show how using an *in vitro* model to study polymorphisms may help us better understand the mechanisms and develop therapeutics for treatment.

5. Methods used to generate mutations to study gene regulation

Identifying genes responsible for different phenotypes previously relied on isolating naturally occurring polymorphic genes within the organisms. This process is time-consuming and requires sequencing several isolates to identify the functionally relevant genes. Today there are many different means of following the general path of altering the genotype to observe the phenotype.

5.1 Site-directed mutagenesis

The use of a point mutation to observe the change of function that occurs as a result of a change in structure underscores the majority of studies on the role of polymorphisms in viral and host gene function. One method of generating a targeted mutation in a plasmid construct carrying the gene of interest uses a polymerase chain reaction (PCR) technique. This particular PCR-based method has the advantage of using a high-fidelity polymerase and methylation of DNA by common strains of *Escherichia coli*. The protocol is described as part of the commercially available QuikChange II™ Site-Directed Mutagenesis Kit (Agilent, 2010). PCR results in the amplification of daughter DNA strands that carry the mutation of interest (Agilent, 2010). In the initial PCR amplification step, two primers containing the mutation of interest bind to the complementary strands of the plasmid. This requires a mismatch of at least one nucleotide on the primers and, furthermore, optimization of the length of the primer for a suitable T^m for the annealing step of the PCR. The mismatch limits

the number of mutations that can be made per reaction to about four bases, depending on the length and T^m of the primer.

PCR will copy the entire parental template. The high-fidelity *Pfu Ultra* polymerase has an error rate of one per 2.5×10^6 nucleotides (Agilent, 2011), making the errors introduced by PCR a non-issue. Once the amplification of the template is complete, the PCR product contains the original parental DNA strands and the mutated and amplified daughter DNA strands. To select against the parental strands, the protocol takes advantage of the fact that the mutated and amplified daughter strands are unmethylated. In contrast, the parental (template) strands are methylated, as they originated from methylation-capable *E. coli*. Digesting the PCR product with *Dpn*I endonuclease, which digests methylated and hemi-methylated DNA, will cleave the parental strands and leave the daughter strands intact. Since the amplified DNA has the plasmid sequence of the parent and since there is an overlap region to allow circularization by homologous recombination, plasmids with the mutation are then isolated by transformation. Any candidate must then be screened and sequenced to confirm the mutagenesis.

6. Structure and function relationship of HIV-1 Vpr

To arrive at the structure of Vpr, several NMR studies have used fragments representing different segments of the Vpr protein. Alternatively the entire Vpr molecule has been analyzed in an appropriate solvent (Morellet et al., 2003; Wecker et al., 2002). The analyses showed a flexible N-terminus with a turn, the first alpha helix, turn, the second alpha helix, turn, the third alpha helix, then a flexible C-terminal domain (Morellet et al., 2003; Wecker et al., 2002). Morellet et al. (2003) used a different solvent, allowing them to "see" the tertiary structure of Vpr. The solvent was better at revealing the hydrophobic parts that are implicated in dimerization and interaction with other proteins (Morellet et al., 2003). Ideally NMR studies of protein structure employ solvents and conditions that approximate physiological conditions. However, the oligomerization property of Vpr makes proper solvation extremely difficult. Several studies have used trifluoroethanol (TFE), a hydrophobic solvent, in different proportions to counteract the interaction of the hydrophobic domains of Vpr, some of which cause Vpr to oligomerize (Engler et al., 2001; Wecker et al., 2002). Across these studies the length of the alpha helices differed due to the proportion of TFE in the solvents used.

Morellet et al. (2003) used CD3CN, a solvent with little hydrophobicity that approaches physiological conditions. This solvent allowed the following structural analysis: alpha helices 1, 2, and 3 (17-33, 38-50, 54-77), a flexible N-terminus (1-16) and a basic C-terminus (78-96). Each helix is amphipathic, containing hydrophobic and hydrophilic residues. The hydrophobic residues can allow for interactions with other proteins, most likely other cellular factors. In the third helix, L60, L67, I74, and I81 can form a leucine zipper (Morellet et al., 2003). The N-terminus consists largely of acidic residues, while the C-terminus consists largely of basic residues, arginine being the most prominent. The 3-D structure established by Morellet et al. (2003; 2009) shows a globular structure, in which hydrophobic interactions between the helices form a lipophilic core. Each of the helices in its amphipathic portions has acidic/basic residues, which are on the external face of the modeled protein. They possibly provide contacts for interactions with other proteins. The folding of the alpha helices entails that hydrophobic portions of the protein face outwards in contact with the

solvent. These unfavorable conditions could be ameliorated by binding to cellular partners. Mutational analyses of the protein have found many residues throughout the length of Vpr that maintain the structural integrity (Morellet et al., 2009). Single deletions of Y15 (see Table 1 in the chapter by Figurski et al. for the amino acid codes), K27, and Q44 result in disruption of structure. The helical domains contain residues crucial to structure, especially those that form the hydrophobic core. The basic C-terminal residues also affect structure and stability of Vpr.

6.1 Oligomerization

The property of forming Vpr oligomers has recently come under scrutiny. Zhao et al. (1994) incorporated mutagenesis in their study to elucidate a rough map of residues in Vpr necessary for this function. Deletion of individual residues in the 36-76 region diminishes formation of oligomers. Mutagenesis of individual resides in the leucine-isoleucine motif (^{60}LIRILQQLLFIHFR) in the third helix (amino acids 55-77) reduced the self-associative capacity of Vpr monomers. The residues at position 60, 61, 63, 64, 67, 68, 70, and 74 mutated from leucine or isoleucine to alanine or histidine disrupted binding between monomers (Zhao et al., 1994). The Q44 residue also plays an important role in Vpr oligomerization. Structural analysis of the second alpha helix (amino acids 38-50) reveals a hydrophilic glutamine at the 44th position (Morellet et al., 2009). Deletion of this residue by site-directed mutagenesis disrupted the secondary structure and abolished Vpr—Vpr interaction (Fritz et al., 2008). Fritz et al. (2008) revealed via 3-D modeling that the ΔQ44 mutation destabilizes the formation of the hydrophobic core and the self-interaction of the helices, thus providing an explanation for the inability of such mutants to oligomerize. Although Vpr oligomerization plays a role in other functions, this group did not find a relation between oligomerization and the ability of Vpr to induce apoptosis.

Oligomerization seems necessary for other functions, such as nuclear localization and virion incorporation (Fritz et al., 2008; Fritz et al., 2010; Venkatachari et al., 2010). Venkatachari et al. (2010) hypothesized from a structural model of the oligomerization of Vpr that residues at the predicted helical interfaces contribute to dimerization. Substitution mutagenesis of A30 to leucine abolished dimerization. The authors attributed this to the position of A30 on the external face of the tertiary structure of Vpr, where it likely affects protein-binding. Furthermore, elimination of dimerization of Vpr abolished the ability of the protein to be incorporated into HIV virions. It also diminished its nuclear localization. These results implicate a necessary role of Vpr oligomerization in its incorporation into virions and, by extension, possibly playing a role in the infection of non-replicating immune cells, important targets of HIV-1.

6.2 Nuclear import

The nuclear import of the preintegration complex (PIC) upon entry of the virus enables productive infection to occur in targeted host cells, particularly non-dividing immune cells (*e.g.*, macrophages/monocytes) (Bukrinsky et al., 1992). As Vpr is bound to the PIC, Vpr shuttles the viral contents into the nucleus, where integration occurs (Popov et al., 1998). An earlier study established the necessity of Vpr in productive viral replication in macrophages (Connor et al., 1995), eventually leading to the identification of importin-α as an essential host factor in this process (Nitahara-Kasahara et al., 2007). Sherman et al. (2001) mapped

non-canonical nuclear localization signals (NLS) throughout the helical domains and the C-terminus of Vpr. This group used site-directed mutagenesis of its various domains in order to identify particular residues that function in nuclear import. In the C-terminus of Vpr, mutagenesis to change several arginine residues (those at positions 73, 76, 77, 85, 87, 88, and 90) to alanine resulted in the distribution of Vpr throughout the cell or cytoplasm, suggesting the importance of this segment of Vpr. In the N-terminus, leucine motifs with the consensus sequence LXXLL in the first and third helices enable nuclear localization. The sequences for each motif are as follows: in the first helix, ^{22}LLEEL^{26}; and in the third helix, ^{64}LQQLL^{68}. The following mutations disrupted nuclear localization most drastically: L22A, L23A, L26A, L64A, and L68A. Via site-directed mutagenesis, the authors established the importance of residues in both helices that are needed for Vpr translocation.

7. G2 cell cycle arrest

The G2 phase of the cellular life cycle serves as a checkpoint for the cell. Factors can halt the cell cycle progression into mitosis in the presence of excessive DNA damage. If these factors do not detect damage, the cell divides; but detection of chromatin disruption will activate factors, such as ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia mutated and Rad-3 related). These factors have downstream effects. Ultimately they hyper-phosphorylate the Cdc2-Cyclin B1 complex, the major controller of cell progression into mitosis. Hyper-phosphorylation inactivates Cdc2-Cyclin B1 to prevent cell division. This process arrests the cell in the G2 phase (Morellet et al., 2009; Pandey et al., 2009; Sherman et al., 2002). Vpr expression in various cell types leads to G2 cell cycle arrest. It does so through increased expression of p21 through the p53 pathway, a major regulator of progression through the G2 and M phases (Chowdhury et al., 2003). Vpr seems to act synergistically with p53, perhaps inducing transcription of p21 through its own transactivation mechanisms. Although the purpose of the G2 arrest function of the virus is debatable, evidence suggests that it enhances transcription from the viral promoter, the long terminal repeat (LTR) of the HIV genome (Goh et al., 1998). One mechanism by which this occurs is through enhancing transactivation of the LTR in CD4+ T-cells via Vpr itself. Vpr binds to other host transcription factors, which bind to sites in the LTR, allowing viral transcription to occur. This effect is enhanced during G2 arrest (Gummuluru and Emerman, 1999).

The C-terminal portion of the protein seems to be essential for induction of cell cycle arrest. Zhou and Ratner (2000) showed that the phosphorylated S79 residue is necessary for this function. Their mutagenesis study of substituting alanine for serine eliminated phosphorylation at this residue and abolished arrest in the G2 phase, correlating the two functions with each other. Furthermore, the mutation of G75A impaired G2 cell cycle arrest, as shown by Mahalingam et al. (1997). DeHart et al. (2007) proposed that Vpr hijacks the ubiquitin/proteasome pathway. One of the functions of this pathway is to target proteins for degradation and to cause G2 cell cycle arrest when needed. In their model, Vpr binds DCAF1, a subunit of the Cullin-4 E3 ubiquitin ligase, to lead to ubiquitination of an unknown host factor involved in halting the cell cycle progression. Change of Q65 to arginine eliminated this binding and impaired G2 cell cycle arrest. However, the group also generated an R80A substitution that also disrupted G2 cell cycle arrest; but this mutant Vpr maintained the ability to bind DCAF1. The authors interpreted these results to indicate that binding DCAF1 is necessary, but not sufficient, to cause G2 cell cycle arrest. A recent study

revealed a highly conserved motif in the C-terminus (^{79}SRIG^{82}), in which mutations that change each amino acid prohibit G2 cell cycle arrest. This study corroborates previous evidence of the functional role of the R80 residue (DeHart et al., 2007; Maudet et al., 2011). Mutagenesis studies using the substitutions R73A and R80A eliminated the induction of p21 transcription, which is necessary for induction of G2 cell cycle arrest.

While the C-terminal portion of Vpr seems necessary for G2 cell cycle arrest, several of the previously cited studies and others revealed that certain N-terminal residues may be indispensable for this function. At least two studies indicated that mutagenesis of A30 to leucine also abolished cell cycle arrest and reduced the transcription of p21 (Chowdhury et al., 2003; Mahalingam et al., 1997). As noted above, A30L eliminated oligomerization, which suggests a correlation between the functions of oligomerization and G2 cell cycle arrest. Also K27M (methionine for lysine 27) another substitution in the first helix, disables the induction of G2 cell cycle arrest. Overall the evidence indicates that N-terminal and C-terminal moieties influence the conformational binding determinants of Vpr involved in G2 cell cycle arrest.

8. Apoptosis

Apoptosis (programmed cell death) functions as a primary means of maintaining homeostasis among cells. Apoptosis can occur as a result of irreparable DNA damage or by disruption of essential cellular processes, such as transcription or translation. Infection by HIV disrupts the normal induction of apoptosis. Despite disrupting apoptosis, infected CD4+ T-cells still die (Groux et al., 1992; Jamieson et al., 1997). However, bystander cells not directly infected by HIV-1, such as CD8+ T-cells, neurons and other cell types, undergo apoptosis (Finkel et al., 1995; Zhang et al., 2003). The Vpr protein was shown to play a key role in the induction of apoptosis in several *in vitro* studies. Vpr was able to permeabilize the mitochondrial membrane and activate caspase 9 via cytochrome C release (Chen et al., 1999; Jacotot et al., 2000; Macreadie et al., 1995; Stewart et al., 1997). Jacotot et al. (2000) established the necessity of several arginine residues inside and between a repeated H(S/F)RIG motif in the Vpr amino acid sequence, specifically ^{71}HFRIG^{75} and ^{78}HSRIG^{82}. Site-directed mutagenesis to generate the substitutions R73A, R77A, and R80A abolished the apoptotic effect of Vpr on Jurkat cells through the pathway involving the mitochondria.

Correlation of the function of Vpr that influences apoptosis with the function that promotes G2 cell cycle arrest remains a complicated affair. Jacquot et al. (2007) reported experiments that indicated a correlation. The group generated several Vpr substitution mutants that abolished both cell cycle arrest and apoptosis. Their results were consistent with the model of G2 cell cycle arrest leading to the induction of apoptosis. Mutants K27M and A30L in the N-terminus of Vpr and R80A and R90K in the C-terminus disabled cytostatic capacity and reduced apoptosis in a T-cell line. However, Bolton and Lenardo (2007) reported that the Vpr with the R80A substitution attenuated apoptotic effects in Jurkat cells, although it remained G2 arrest-capable. Also Maudet et al. (2011) showed that the apoptotic function of alanine mutants in the ^{79}SRIG^{82} motif, including R80, and the K27M substitution mutant remained intact without G2 cell cycle arrest. The evidence from such mutagenesis studies may indicate that, although G2 cell cycle arrest leads to apoptosis, a G2 cell cycle arrest-independent apoptotic pathway exists.

Interestingly, Maudet et al. (2011) showed that the ability of Vpr to bind DCAF1 is not only necessary for induction of G2 cell cycle arrest, but for apoptosis as well. The substitution mutant Q65R eliminated DCAF1 binding, abolishing cell death. The S79A and K27M mutants retained their ability to cause apoptosis while losing G2 arrest function. Mutagenesis was done to produce the double mutants K27M/Q65R and S79A/Q65R. These mutants eliminated the G2 cell cycle arrest-independent induction of apoptosis. As DCAF1 is essential for the targeting of proteins to the proteasome, the authors propose a model in which Vpr binds DCAF1 at a region containing the Q65 residue and functions as an adaptor to the ubiquitin/proteasome complex. Their mutagenesis studies suggest that Vpr contains two different binding domains that interact with two separate, and as yet unidentified, host targets. This ostensibly leads to ubiquitination and subsequent degradation of these proteins. Degradation of target 1 leads to G2 cell cycle arrest, and ubiquitination of target 2 leads to apoptosis. However, these targets have yet to be identified. The model implies complex pathways.

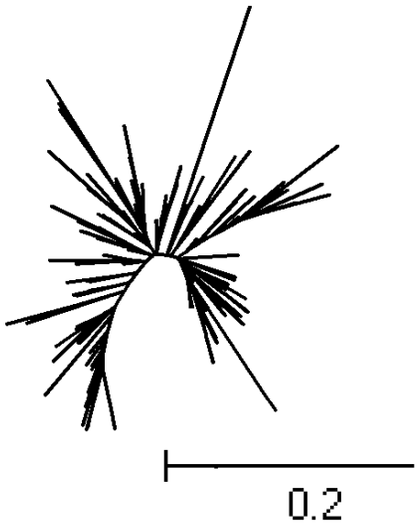

Fig. 2. Phylogeny of *vpr* Sequences Across Clades

9. Vpr polymorphisms across subtypes

A phylogenetic analysis of *vpr* sequences from clades A-D (including the highly prevalent subtype B shows a large diversity of sequences across the genetic lineages of HIV-1 subtypes **(Figure 2).** This indicates the existence of quasispecies of Vpr existing in the population and selective pressures acting on the *vpr* gene. Upon closer analyses of the sequences within the tree, several of the species between subtypes of Vpr show closer genetic relationships than other species within subtype. The variations in Vpr may correlate more to disease progression than to the clades. A comparative analysis of the frequencies of *vpr* alleles from long-term non-progressors (LTNP) and rapid progressors (RP) indicates the presence of mutations that are of interest **(Table 1)**.

Position	Consensus Sequence of LTNP alignment Residue	Residue conserved In the LTNP alignment (n=177)	Residue In Consensus Seq PR alignment	Residue conserved In the PR alignment (n=102)
2	E	174	E	101
3	Q	168	Q	96
4	A	175	A	96
5	P	176	P	101
6	E	166	E	100
7	D	170	D	91
8	Q	175	Q	101
9	G	165	G	102
10	P	176	P	102
11	Q	174	Q	98
12	R	167	R	102
13	E	168	E	100
14	P	177	P	100
15	Y	172	Y	96
16	N	158	N	96
17	E	169	E	96
18	W	175	W	102
19	T	153	T	93
20	L	173	L	102
21	E	174	E	102
22	L	170	L	95
23	L	170	L	102
24	E	169	E	100
25	E	172	E	102
26	L	173	L	101
27	K	172	K	102
28	N	74	S	40
29	E	171	E	101
30	A	170	A	102
31	V	171	V	101
32	R	167	R	100
33	H	174	H	101
34	F	174	F	101
35	P	175	P	101
36	R	171	R	88
37	I	50	V	51
38	W	174	W	101
39	L	175	L	99
40	H	175	H	88
41	S	89	G	71
42	L	171	L	101
43	G	171	G	101

Position	Consensus Sequence of LTNP alignment Residue	Residue conserved In the LTNP alignment (n=177)	Residue In Consensus Seq PR alignment	Residue conserved In the PR alignment (n=102)
44	Q	175	Q	101
45	H	118	H	72
46	I	174	I	101
47	Y	173	Y	101
48	E	146	E	92
49	T	174	T	98
50	Y	175	Y	101
51	G	169	G	101
52	D	175	D	100
53	T	175	T	100
54	W	174	W	98
55	A	123	A	58
56	G	171	G	100
57	V	174	V	100
58	E	164	E	97
59	A	166	A	96
60	I	149	I	62
61	I	167	I	96
62	R	170	R	96
63	I	136	I	53
64	L	176	L	99
65	Q	173	Q	97
66	Q	174	Q	96
67	L	174	L	98
68	L	174	L	82
69	F	176	F	100
70	I	145	I	93
71	H	173	H	100
72	F	174	F	99
73	R	174	R	100
74	I	172	I	97
75	G	158	G	95
76	C	171	C	99
77	R	106	R	52
78	H	174	H	100
79	S	175	S	98
80	R	172	R	99
81	I	171	I	97
82	G	174	G	100
83	I	171	I	99
84	T	99	T	66

Position	Consensus Sequence of LTNP alignment Residue	Residue conserved In the LTNP alignment (n=177)	Residue In Consensus Seq PR alignment	Residue conserved In the PR alignment (n=102)
85	R	111	Q	40
86	Q	143	Q	83
87	R	168	R	94
88	R	168	R	97
89	A	140	A	83
90	R	169	R	95
91	N	170	N	56
92	G	171	G	57
93	A	153	A	55
94	S	162	S	51
95	R	173	R	58†

Table 1†. Frequency Analysis of Amino Acids Resulting from *vpr* Alleles Found in the LTNP and RP Groups

An approach using site-directed mutagenesis to study these substitutive polymorphisms will link these variants to possible effects on Vpr function and to the long-term non-progressor and rapid progressor statuses.

10. Mutagenesis studies in the context of disease progression and pathogenesis

Wang et al. (1996) analyzed sequences of the *vpr* genes from an HIV-infected mother-child pair who showed no sign of AIDS from initial infection in 1983 to the time of the study in 1995. These investigators found that samples from the mother and child had homogeneous and similar length polymorphisms in the C-terminal region of Vpr. However these polymorphisms were not present in samples from 30 patients who developed AIDS. Several other studies showed marked heterogeneity in *vpr* sequences derived from multiple patient samples (Ge et al., 1996; Kuiken et al., 1996). In addition to length polymorphisms, numerous studies have demonstrated an association between substitution mutants in the amino acid sequence of Vpr and disease (Caly et al., 2008; Lum et al., 2003; Tungaturthi et al., 2004).

Caly et al. (2008) found a mutation, F72L, in several *vpr* sequences derived from a single patient with LTNP status. This mutation seems to be correlated with the disruption of nuclear import. However only a limited conclusion about prognosis can be drawn from this result because of the small sample size. Yedavalli and Ahmad (2001) reported several mutations from *vpr* sequences extracted from HIV-infected mothers with LTNP status who did not transmit the infection to the child during labor. They found polymorphisms from two separate patients that led to the A30S and G75R substitutions in Vpr. They also found

† Amino acid abbreviations follow standard conventions from the International Union of Pure and Applied Chemistry (IUPAC) (Nomenclature, 1968).

C-terminal deletions at an abundant frequency in another sample. This finding implies the possibility of structural changes to Vpr that might alter function in the context of disease progression. The involvement of Vpr at all stages of the HIV-1 life cycle suggests that this protein influences the development of disease and the severity of the outcome. Our hypothesis is that with a sufficient sample size, assessment of *vpr* sequences derived from the LTNP and RP groups will reveal signature polymorphisms that may be linked to progression of AIDS *in vivo*. This may occur through the disruption or enhancement of hallmark Vpr functions. Already several groups have suggested that unique, signature polymorphisms in Vpr culled from LTNP patient samples are associated with the reduction of host cell apoptosis (Lum et al., 2003; Somasundaran et al., 2002).

However, given the complexities of the interrelated pathways by which Vpr induces apoptosis, as revealed by the mutagenesis studies detailed above, it is likely that the two published studies explain only a fraction of the causative factors behind the progression of HIV disease. Many questions need to be answered before the role of Vpr in disease induction is clear.

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